

SEASONAL CHANGES IN THE RUMEN MICROBIAL ECOLOGY OF MUSKOXEN

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SEASONAL CHANGES IN THE RUMEN MICROBIAL ECOLOGY OF MUSKOXEN

A

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By

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ABSTRACT

Ruminal fermentation must respond to seasonal changes in the behavior and digestive function of muskoxen (*Ovibos moschatus*) as intakes of food and water rise in autumn (August – October) and decline from winter to spring (November – May). Rumen temperatures were not static but instead were punctuated by decreases of as much as 13°C below the average of 39°C. Declines in rumen temperature were associated with water ingestion while gains in rumen temperature were associated with feeding. Food intake and water turnover rates were positively correlated. Direct microscopic counts of bacteria in rumen fluid decreased from 1.8 to $0.9 \times 10^{10} \cdot \text{mL}^{-1}$ as water turnover declined from 11.1 to $7.7 \text{ kg}^{-1} \cdot \text{d}^{-1}$ between October and March. The energetic cost of warming ingesta was estimated as 5 to 14% of the predicted intake of digestible energy. Concerted ingestion of food and water may allow muskoxen to substitute heat increment of feeding for the cost of warming ingesta. Gains in food intake between May and August decreased rumen osmolality (271.9 ± 16.4 vs. $245.9 \pm 11.4 \text{ mOsmol} \cdot \text{kg}^{-1}$) and pH (6.81 ± 0.31 vs. 6.39 ± 0.15), indicating a shift in the allostatic set point for the rumen. Rumen fluid pH was more variable in May than in August both before and after a single meal of fermentable substrate even though concentrations of fermentation acids were lower in May than in August. Allostatic set points probably alter the homeostatic range of conditions and the microbial diversity of fermentation in muskoxen. We devised an improved method to isolate rumen bacteria and to extract their DNA for measures of rumen microbial diversity.

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INTRODUCTION

Ruminants rely on symbiotic microorganisms within the foregut to degrade their fibrous forage. Microbial fermentations within the foregut are favored by stable rumen conditions of temperature, osmolality, and pH (Hungate 1966). However, disturbances to rumen homeostasis may impair fiber degradation by the microbial community and subsequently alter the quantity of fermentation products available to the host animal (Dehority 2003). Food intake by the ruminant dictates the quantity of substrate available for microbial fermentation. Ruminants from northern latitudes exhibit wide fluctuations in food intake due to seasonal availability of forage. Seasonal shifts in food intake by arctic grazers cause potential disruptions to the microbial ecosystem, causing changes in substrate load as well as potential fluctuations in rumen environmental conditions.

Muskoxen (*Ovibos moschatus*) are large ruminants that forage in polar regions throughout winter when access to food is limited. However, food intake increases by 74% in muskoxen from winter and spring to autumn with the emergence of new forage, imposing greater substrate loads on the microbial population (Barboza *et al.*, 2006). High food intakes correspond to declines in rumen fluid pH and increases in short chain fatty acid (SCFA) concentrations in muskoxen (Barboza *et al.*, 2006). Nevertheless, it is unclear whether rumen conditions such as temperature, osmolality, and pH vary in response to natural fluctuations in food intake or supplementation with an acute load of fermentable carbohydrates. Because changes in fermentable substrate load and conditions within the rumen may adversely affect microbial activity, the energy supply to the host animal could likewise be impaired.

In particular, ingestion of cold food or water at low ambient temperatures (*e.g.*, -40°C) may affect microbial activity by decreasing the concentrations of bacteria and their fermentation products (SCFA) within the rumen (Hungate 1966; Roger *et al.*, 1990). When the rumen is dosed with cold water or food, energy expenditures by the animal increase (Blaxter 1962). However, we do not know the effect of natural ingestive behaviors on rumen temperature, water flux, bacterial concentrations, or the thermal costs of over-wintering ruminants.

Rumen environmental parameters may change not only during periods of low food intake throughout winter and spring but also during periods of high food intake in autumn. The rumen microorganisms within the foregut of muskoxen may be exposed to changing conditions of temperature, osmolality, and pH. Fluctuation in food intake by the host animal may tax the cellulolytic abilities of the microbial population. Moreover, concentrations of bacteria and SCFA in these arctic ruminants may fluctuate, or the microbial population structure itself may change in response to both high and low food intake. Therefore, estimates of rumen conditions as well as microbial diversity are important in determining the extent and efficiency of cellulose degradation within the foregut.

In the present study, we examined the effects of seasonal shifts in food intake as well as supplementation on rumen environmental parameters, concentrations of bacteria and fermentation acids, and the energetic costs to the host. We also introduce a novel DNA isolation and extraction technique to assess rumen microbial diversity in heterogeneous rumen digesta samples.

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CHAPTER 1. THE RUMEN IN WINTER: COLD SHOCKS IN NATURALLY FEEDING MUSKOXEN (*OVIPOS MOSCHATUS*)¹

ABSTRACT. — We fed castrated adult muskoxen (*Ovibos moschatus*) a consistent diet throughout winter to relate ambient conditions and ingestive behavior to rumen temperature, water flux, and the number of bacteria within the rumen during October, January, and March. Captive muskoxen in Fairbanks, AK, USA (65°N, 146°W) were exposed to air temperatures as low as -40.1°C with minimum daylengths of 2.9 hrs in winter. Rumen temperatures were not static but instead were punctuated by decreases of as much as 13°C below the average of 39°C. Declines in rumen temperature were associated with water ingestion, while gains in rumen temperature were associated with feeding. Food intake and water turnover rates were positively correlated. Water turnover rates were high in October (11.1 kg⁻¹·d⁻¹) and low in January and March (9.8 and 7.7 kg⁻¹·d⁻¹), respectively. Numbers of bacteria in rumen fluid decreased with water turnover and thus food intake from October (1.8 X 10¹⁰·mL⁻¹) to January and March (0.94 and 0.88 X 10¹⁰·mL⁻¹). Muskoxen spent more time consuming water as snow than as free water, which may have reduced the thermal shock of water ingestion in winter. The energetic cost of warming ingesta was estimated from water turnover rates at 25, 79, and 57 kJ·kg^{-0.75} in October, January, and March, respectively, and was 5 to 14% of the

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predicted intake of digestible energy. Simultaneous ingestion of food and water may allow muskoxen to substitute heat increment of feeding for the cost of warming ingesta. In muskoxen, rumen fermentation apparently can tolerate large cold shocks but changes in food intake probably dictate the size of the microbial community.

INTRODUCTION

Mammals and birds defend large differences in temperature between ambient conditions and their body core when living in polar regions (Blix, 2005). Heat loss from the animal's surface increases with lower ambient temperatures whereas heat loss from within the animal increases with the ingestion of cold water or food. Muskoxen (*Ovibos moschatus*) are large grazing ruminants that forage throughout winter when ambient temperatures are often consistently below -40°C. The fibrous nature of their diet limits the energy available for thermal response. Muskoxen also reduce their energy expenditures (Lawler and White, 2003) and ingestion rates of food (Peltier et al., 2003) and water (White et al., 1987) during winter. However, we do not know if changes in feeding and drinking rates offset the potentially large costs of heating ingesta during winter.

Ingestion of cold food or water may adversely affect microbial fermentation in the rumen. Fermentation accounts for 79% of dry matter digestion in muskoxen and is thus the principal source of energy for the animal (Barboza et al., in press). Muskoxen can use short chain fatty acids (SCFA) as well as the microbial cells produced in the rumen for energy. Cold shocks may decrease microbial activity, reducing both the number of microbes as well as the production of SCFA (Hungate, 1966). Adhesion of rumen bacteria (e.g., *Fibrobacter succinogenes*) to fibrous substrates is optimal at 38°C, while lower or higher temperatures markedly reduce attachment (Roger et al., 1990). Therefore, deviations in rumen temperature may impair fiber digestion and thus the energy supply to the animal.

It has long been assumed that conditions within the rumen are relatively constant, because rumen microbes are favored by a stable environment (Russell, 2002). Core body temperatures of ruminants are remarkably stable throughout the year (Fuller et al., 2005). Variable or low body temperatures preclude microbial activities such as ureolysis in wintering heterotherms and ectotherms (Banas et al., 1988; Barboza et al., 1997). Dosing the rumen of domestic animals with chilled water and food dramatically reduces temperature of digesta (Degan and Young, 1984; Nicol and Young, 1990) and increases energy expenditure (Blaxter, 1962). However, we do not know how natural foraging behaviors affect rumen temperature and thermal costs of over-wintering ruminants. The objective of this study was to evaluate the thermal cost of extreme winter conditions on captive muskoxen provided a consistent diet. We assessed animal behavior and ambient conditions throughout winter to determine the influence of consuming cold forage and water or snow on rumen temperature, water flux, and the number of bacteria within the rumen.

MATERIALS AND METHODS

Animals & Behavior. —

We studied 4 castrated adult (6.4 to 9.5 yrs) muskoxen (*Ovibos moschatus*) with ruminal fistulas at the R. G. White Large Animal Research Station of the University of Alaska Institute of Arctic Biology (Fairbanks, Alaska; 65°N, 146°W). Animals were surgically cannulated 3.5 years prior to the study. All experimental procedures followed the American Society of Mammalogists guidelines and were approved under protocol #03-21 by the Institutional Animal Care and Use Committee, University of Alaska, Fairbanks.

Experiments were conducted during the following seasons in 2003 to 2004: early winter (October), mid winter (January), and late winter (March).

Animals were marked with nontoxic spray paint for ease of identification during behavioral studies. Muskoxen were housed in a large fenced enclosure (2,043 m²) with ad libitum access to grass hay (*Bromus* sp.) and water or snow. A pelleted mixture of cereal grains (M Ration, Alaska Pet and Garden, Anchorage, Alaska) was offered on an individual basis, twice a week, as a supplement.

Animal behaviors including eating, ruminating, fighting, running, walking, standing, lying, and drinking water or ingesting snow were monitored (LARS Behavioral Program; W.E. Hauer, personal communication) for two mornings (10:00 to 14:00; Alaska Standard Time) and two afternoons (13:00 to 17:00) in each month to correlate animal behavior with rumen temperature patterns. Body mass was recorded (± 0.5 kg Tru-Test Model 703 scale, San Antonio, Texas) pre and post observational period and during dosing and bleeding of ³H₂O to monitor body condition. Animals were trained to handle mild physical restraint in a squeeze chute for weighing and bleeding purposes without the use of sedatives or chemical immobilants.

Rumen Temperatures and Ambient Conditions. —

HOBO Water Temp Pro Data Loggers (Onset Computer Corp., Bourne, Massachusetts) were tethered to each rumen cannulae, allowing for full rotational motion, and recorded rumen temperature to an accuracy of 0.03°C every 30 seconds for 8 days in each study period. Ambient conditions including air, water, snow, and hay temperatures (12-Bit

Temperature Smart Sensor, accuracy: $\leq \pm 0.2^{\circ}\text{C}$ from 0° to $+50^{\circ}\text{C}$, measurement range: -40° to $+75^{\circ}\text{C}$) as well as wind speed (Wind Speed Smart Sensor, accuracy: $\pm 1.1 \text{ m}\cdot\text{sec}^{-1}$, measurement range: 0 to $45 \text{ m}\cdot\text{sec}^{-1}$) and solar radiation (Silicon Pyranometer Smart Sensor, accuracy: $\pm 10 \text{ W}\cdot\text{m}^{-2}$, measurement range: 0 to $1280 \text{ W}\cdot\text{m}^{-2}$) were simultaneously measured (HOBO Weather Station, Bourne, Massachusetts) and pooled in 10 min intervals to correlate environmental conditions with rumen temperature patterns.

Chemical Analyses. —

Common hay and individual supplement provided to muskoxen were sampled every 3 days and dried at 50°C for 48 hrs to determine dry matter. Foods were ground through 2mm mesh in a Wiley mill (Arthur Thompson Company, Philadelphia, Pennsylvania) for further analysis. Analytical dry matter was determined by drying to constant mass at 80°C . Ash content was measured by combusting 1 g of dried material for 8 hrs at 500°C in a muffle furnace (Barnstead International, Dubuque, Iowa). Plant cell wall contents were extracted sequentially as follows: neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin. Fiber extractions followed the procedures of Peltier et al. (2003). Hemicellulose content was calculated as the difference between NDF and ADF whereas cellulose content was calculated as the difference between ADF and lignin (Van Soest et al., 1991; Peltier et al., 2003).

Water Turnover Rates. —

Water flux in animals was determined by tritiated water ($^3\text{H}_2\text{O}$) dilution following an intrajugular dose of $1.80 \mu\text{Ci}\cdot\text{kg}^{-1}$ body mass in 0.9% NaCl solution (Sigma Chemical Company, St. Louis, Missouri; Peltier and Barboza, 2003). Blood was collected in Na-Heparin evacuated tubes (Vacutainer Systems, Becton Dickinson, Franklin Lakes, New Jersey) from the jugular vein prior to dosing and at 6, 48, 96, 168, and 216 hrs post dose. Heparinized blood was transferred to capillary tubes and spun in an IEC Micro-MB centrifuge (International Equipment Co., Needham Heights, Massachusetts) for 5 minutes at high speed to determine hematocrit. Plasma was separated from heparinized blood by centrifugation at $1000 \times g$ for 10 minutes and stored at -20°C for analysis.

Tritium concentrations in plasma were determined by scintillation counting and corrected for background and quenching (Beckman LS6000SE, Beckman Instruments Inc., Redmond, Washington). The least squares regression of plasma tritium counts over time ($\text{Ln} [\text{H}^3]$) was used to calculate body water kinetics. Assuming that ingesta comprise 16.02% body mass (BM) with 0.886 g H_2O per g ingesta (Barboza et al, in press), ingesta free mass (IFM) was calculated as $\text{IFM} = \text{BM} - (\text{BM} \times 0.1602)$. Net water space (NW) was calculated from water space (W) as $\text{NW} = (\text{W} \times 0.9) - (0.886 \times 0.1602 \times \text{BM})$ and was adjusted by 10% to correct for the overestimation of tissue water pool (Chan-McLeod et al., 1994). Lean mass (NM) was calculated as 73.3% water (Adamczewski et al., 1995) or $\text{NM} = \text{NW} \div 0.733$. Lipid mass (LM) was then calculated as the difference between lean and ingesta free mass: $\text{LM} = \text{NM} - (0.1602 \times \text{BM})$.

Rumen Sampling. —

In each month, 2 X 30 mL whole digesta samples were collected from each animal by drawing from the top of the raft with forceps. We extracted 100 additional mLs of rumen digesta and measured pH with a standardized electrode (± 0.01 units) within 5 minutes of sample collection. Rumen digesta were then strained through sterile, 12-ply, USP Type VII gauze (Johnson & Johnson, New Brunswick, New Jersey). Aliquots of 15 mL rumen fluid were transferred to equal volumes of 10% formalin for bacterial counts. Bacteria were counted by phase contrast microscopy (Olympus Optical Co., Japan) and a hemocytometer (Bright Line Counting Chamber, Hausser Scientific, Horsham, Pennsylvania). All formalin samples were diluted to provide 40 to 70 bacteria per cell.

Statistical Analyses. —

We conducted statistical analyses in Systat 10.2 (SPSS Inc., Chicago, Illinois). Repeated measures of body composition, rumen temperature and ambient conditions, bacterial counts, and rumen fluid pH were compared by ANOVA with winter time period as a factor ($n = 3$) within animals. Summary statistics were calculated based on rumen temperature and ambient condition data. Statistical significance was determined at $\alpha < 0.05$.

RESULTS

Muskoxen experienced the lowest air temperatures during mid winter (January) when solar radiation was at the annual nadir (Table 1.1). Wind speeds were low throughout

winter as is typical for interior Alaska (Table 1.1). The mean ambient temperature for January was -29°C with a range of -41° to $+17^{\circ}\text{C}$ throughout the study. Temperatures of dry hay followed the pattern of ambient air temperatures and were therefore lowest in January. Water was available as liquid only in October and as snow for the remainder of the study until the following May (Table 1.1).

Average rumen temperatures were similar among periods in winter (Table 1.2). Maximal rumen temperatures were less than 1°C above the average temperature of the rumen. Minimal rumen temperatures were variable and were often reached after rapid drops at rates of up to $6.24^{\circ}\text{C}\cdot\text{min}^{-1}$ to minima of 27.6°C (Table 1.2, Fig. 1.1A-C). Following cooling events, rewarming of the rumen was slower than cooling, as rates of temperature rise were always less than rates of decline.

Fluctuations in rumen temperature were not associated with changes in the temperature of ambient air, hay, or available water. Some acute drops in rumen temperature were associated with drinking water or ingesting snow whereas some gains in rumen temperature were associated with feeding bouts (Fig. 1.1D-F). Feeding rate did not vary between periods of observation during winter ($0.22 \pm 0.04 \text{ min}\cdot 100 \text{ min}^{-1}$ observed; $F_{2,9} = 1.3575$; $P = 0.31$). The proportion of time spent consuming water as liquid (October) was less than that required for snow ingestion (January and March; Fig. 1.2). Drinking behavior was positively related to time spent feeding whether the water was available as liquid or as snow (Fig. 1.2). Time spent on behaviors other than ingestion (e.g., lying, ruminating, walking, standing, running, fighting) did not differ between periods ($P > 0.05$).

Total turnover of water in muskoxen was greatest in early winter and lowest in late winter (Table 1.3). Muskoxen lost body mass through winter even though diets did not change in composition (Tables 1.3 & 1.4). Water space and thus lean mass were significantly lower in March than in October ($P = 0.02$) whereas body fat was highest in March. Changes in water space and body composition were not related to changes in hematocrit which were consistent through winter (45.2 to 45.9%). Numbers of rumen bacteria decreased with water turnover rates from October to January ($P = 0.01$) and remained low in March (Fig. 1.3). Changes in numbers of rumen bacteria were not associated with rumen pH, which did not significantly vary over winter (6.85 ± 0.11 ; $P = 0.41$).

DISCUSSION

Muskoxen experienced air temperatures as low as -40.9°C but maintained an average rumen temperature of $+38.8^{\circ}\text{C}$, to produce a maximum thermal difference of 79.7°C . Wild muskoxen likely defend even greater thermal differences in the high arctic where air temperatures may drop below -60°C (Blix, 2005). With such large thermal differences, we may anticipate that muskoxen incur considerable thermoregulatory costs when warming frozen food and water to rumen temperature.

The thermal cost associated with warming water was estimated from water turnover rates (Table 1.3) and predicted food intakes. We assumed that seasonal intakes of food followed the pattern previously measured in castrated muskoxen by Peltier et al. (2003). Intakes of dry hay in October were equated to the annual maxima ($53 \text{ g}\cdot\text{kg}^{-1}$

$^{0.75} \cdot \text{d}^{-1}$) whereas intakes in January and March were equated to the annual minima ($31 \text{ g} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$). Water ingested with food was calculated from the moisture content of hay (Table 1.4). Free water intakes were subsequently derived by subtracting food water from total water turnover. Warming costs were estimated from constants for warming ice (specific heat of ice; $2.1 \text{ J} \cdot \text{g}^{-1} \cdot ^\circ\text{C}^{-1}$), melting ice (latent heat of fusion; $335 \text{ J} \cdot \text{g}^{-1}$), and warming water (specific heat of water; $4.184 \text{ J} \cdot \text{mL}^{-1} \cdot ^\circ\text{C}^{-1}$) in free water and food (Weast, 1973). The cost of warming dry food was calculated with the specific heat for cellulose ($1.3 \text{ J} \cdot \text{g}^{-1} \cdot ^\circ\text{C}^{-1}$; Berteaux, 2000). We equated daily intakes of digestible energy (DEI) in early winter to the annual maximum ($923 \text{ kJ} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$) whereas intakes in mid and late winter were equated to the annual minimum ($554 \text{ kJ} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$) as determined by Peltier et al. (2003). Thermal costs were calculated for food and water ingested at the average air temperature in each period (Table 1.1) and warmed to the corresponding average rumen temperature (Table 1.2).

Total costs of warming food and water were 25, 79 and $57 \text{ kJ} \cdot \text{kg}^{-0.75}$ in October, January, and March, respectively. Water was consumed as ice in January and March but as liquid water in October. The cost of melting ice in all ingesta (latent heat of fusion) was much greater than the incremental cost of increasing temperature to either liquid or ice. Consequently, melting frozen ingesta accounted for 58 to 63% of the total cost of warming during mid to late winter.

Energy used to warm ingesta is primarily derived from the digestible energy intake of the animal. Warming costs are equivalent to 2.7, 14.2, and 10.3% of the digestible energy intake in early, mid, and late winter when diet quality is maintained.

The proportional cost of warming ingesta is dependent on both rate of food intake and diet quality. Proportional costs of warming ingesta may be insensitive to differences in body size and total energy intakes (Berteaux, 2000) because water intakes follow food consumption. Food intake may, however, increase more rapidly than water intake if the digestible energy content of the diet declines. Therefore, increased consumption of less digestible foods may exacerbate the proportional cost of warming and thus reduce the net return of energy to the animal. The broad range of estimates for warming ingesta (2.5 to 30% of daily energy expenditure; Berteaux, 2000) reflects both the large variation in solute loads and digestibility of diets across a wide range of body sizes in over-wintering ruminants.

The cost of warming ingesta may be compensated by the heat increment of feeding. Lawler and White (2003) estimated heat increments of feeding that were $58 \text{ kJ} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$ at peak food intakes but only $39 \text{ kJ} \cdot \text{kg}^{-0.75} \cdot \text{d}^{-1}$ in winter. Heat increments of feeding exceed the cost of warming ingesta in October and were at least 50% of the lowest increment in January and March. Close association of feeding and drinking behavior would limit changes in rumen temperature and the cost of warming water after feeding (Fig. 1.1D-F). Feeding and drinking behaviors have been linked by specific sensory properties of food that simulate drinking in ruminants (Langhans et al., 1995). The positive relationship between time spent drinking and feeding in muskoxen is consistent with this suggestion of linked behaviors (Fig. 1.2). The rate of water entering the rumen in winter is reduced by the consumption of snow instead of liquid water. Ingestion of snow requires proportionately more handling time in relation to food intake.

Consequently, the thermal load of drinking is more gradual in winter because muskoxen spend more time ingesting less water.

The thermal environment of the rumen is generally assumed to vary by a range of only 2°C in domestic species (Dehority, 2003; Hungate, 1966). The thermal stasis of the rumen (Forbes and France, 1993) is probably an outcome of linked feeding and drinking in animals grazing naturally. However, acute doses of cold water to the rumen can drop rumen temperature by 5 to 10°C in domestic cattle and sheep (Dehority, 2003; Cunningham et al., 1964). In contrast, voluntary feeding and drinking in muskoxen produces random cold shocks to the rumen that are similar to the acute doses of water in domestic animals.

Rumen fermentations in muskoxen apparently tolerate abrupt declines in temperature because the rumen requires several hours to rewarm. Cold shocks may impair substrate binding of bacteria, for example, binding of *Fibrobacter succinogenes* to cellulose is reduced by 14% when temperature drops by 13°C (Roger et al., 1990). Thus, natural drops in the rumen temperature of muskoxen may simply impair microbial activity by dislodging bacteria from their substrates (Olubobukun et al., 1988) and by reducing enzyme activity. Cold shocks may therefore reduce SCFA production for the animal until the rumen rewarms. Conversely, rumen temperature apparently never exceeds an upper limit of 41°C in muskoxen. Bacterial adhesion to substrates may be much more sensitive to heating above 39°C because a rise of 9°C reduces binding of *F. succinogenes* to cellulose by 60% (Roger et al., 1990). The upper limit of rumen temperature is probably an outcome of the body temperature conducted from the blood

and the optimal temperature for the microbes. Overheating may therefore endanger rumen fermentation more severely than cold shocks, especially when animals are hyperphagic in summer.

Declining numbers of rumen bacteria during winter are probably not related to rumen temperature drops because temperature changes in the rumen were similar among periods in winter (Table 1.2). Small bacterial populations may be an outcome of rumen dilution or low rates of microbial division and activity. Dilution of microbes is unlikely because rumen fill declines by 45% between spring and winter in muskoxen (Barboza et al., in press). In muskoxen, *in situ* degradation of cellulose and other substrates in the rumen is lowest in winter when numbers of bacteria are likewise low in muskoxen (Barboza et al., in press). The availability of substrates for fermentation may ultimately limit microbial populations in muskoxen when food intakes are low in winter. Declines in water turnover rates during winter are consistent with drops in food intake and the diminution of bacterial counts. Seasonal changes in concentrations of fermentation acids that accompany changes in food intake could be associated with changes in the microbial population and shifts in the optima for rumen conditions and substrate availability (Barboza et al., in press).

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Table 1.1. Ambient conditions for muskoxen at Fairbanks, Alaska during winter of 2003-2004. Study periods were 8 days during early (October), mid (January), and late (March) winter. Mean and range (minimum — maximum) for each parameter were derived from records averaged every 10 minutes during each study period.

Parameter	October	January	March
Air (°C)	+2.89 (-2.04 — +16.77)	-28.52 (-40.94 — -19.06)	-6.80 (-27.15 — + 6.00)
Wind Speed (m·sec ⁻¹)	0.41 (0.00 — 2.66)	0.37 (0.00 — 2.66)	0.69 (0.00 — 3.05)
Solar Radiation (W·m ⁻²)	48.6 (0.6 — 429.4)	2.9 (0.6 — 34.4)	88.0 (0.6 — 386.9)
Hay (°C)	+3.84 (-1.58 — +20.22)	-26.70 (-40.94 — -16.08)	-6.43 (-27.09 — -11.01)
Water/Snow (°C)	+3.54 (-0.12 — +13.62)	-21.56 (-28.21 — -15.91)	-8.26 (-20.69 — -0.65)

Table 1.2. Rumen temperature (°C) in muskoxen (n = 4) during early (October), mid (January), and late (March) winter. Temperatures were recorded every 0.5 min for 8 days in each period. Each parameter (mean \pm SD) was not significantly different among periods (ANOVA; $P > 0.05$).

Parameter	October	January	March
Mean	38.86 \pm 0.09	38.85 \pm 0.10	38.70 \pm 0.10
Maximum	39.79 \pm 0.21	39.65 \pm 0.60	39.75 \pm 0.08
Minimum	31.40 \pm 3.78	35.65 \pm 2.24	32.52 \pm 3.30
Maximum Rise (min ⁻¹)	1.26 \pm 0.82	0.32 \pm 0.16	1.64 \pm 0.90
Maximum Drop (min ⁻¹)	4.14 \pm 2.10	1.08 \pm 0.56	3.04 \pm 0.98

Table 1.3. Body mass, water dynamics, and estimated body composition of muskoxen (n = 4) during early (October), mid (January), and late (March) winter (mean \pm SD)¹.

Parameter	October	January	March
Water Turnover (kg·day ⁻¹)	11.1 \pm 1.17 ^A	9.8 \pm 1.05 ^{AB}	7.7 \pm 2.12 ^B
Water Space (kg)	153.38 \pm 7.58 ^A	132.16 \pm 10.88 ^{AB}	123.52 \pm 8.27 ^B
Body Mass (kg)	308.3 \pm 13.12 ^A	300.4 \pm 12.0 ^B	295.8 \pm 11.97 ^C
Lean Mass (kg)	209.26 \pm 10.36 ^A	180.30 \pm 14.85 ^{AB}	168.51 \pm 11.29 ^B
Fat (kg)	49.4 \pm 9.09 ^A	74.6 \pm 24.28 ^{AB}	79.4 \pm 7.52 ^B

1. Different superscripts indicate significant pair-wise differences among periods within each parameter.

Table 1.4. Mean composition of dry matter in hay and supplement fed to fistulated muskoxen during early (October), mid (January), and late (March) winter.

Component (g·g ⁻¹)	Hay			Supplement		
	October	January	March	October	January	March
Dry Matter	0.95	0.94	0.97	0.94	0.95	0.97
NDF	0.57	0.67	0.60	0.31	0.31	0.30
Hemicellulose	0.26	0.28	0.28	0.16	0.16	0.15
ADF	0.30	0.38	0.33	0.15	0.18	0.15
Cellulose	0.26	0.33	0.28	0.08	0.15	0.13
Lignin	0.04	0.05	0.04	0.02	0.03	0.03
Ash	0.07	0.04	0.05	0.09	0.09	0.09

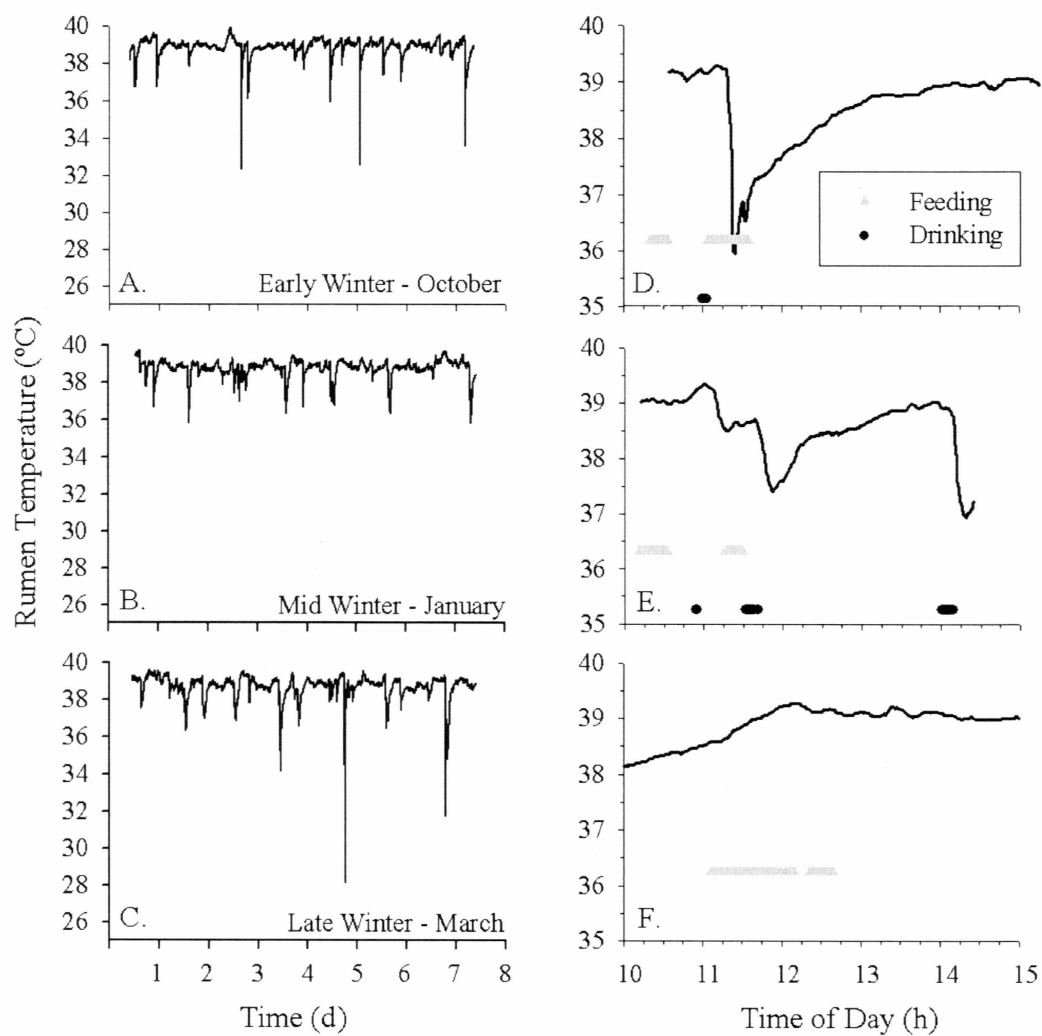


Figure 1.1. Representative records of rumen temperature in muskoxen. Records are plotted over 8 days in early (A), mid (B) and late (C) winter. Rumen temperatures during behavioral observations are plotted in each winter period (D-F).

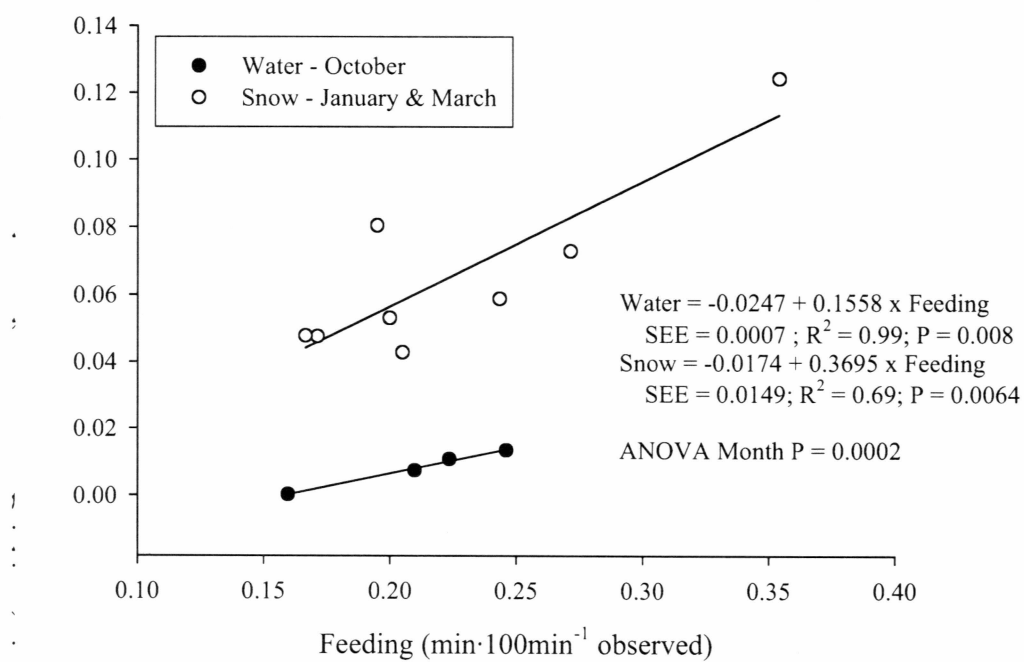


Figure 1.2. Relationships between time spent drinking and feeding in muskoxen when water is available as liquid in early winter (October) and as snow in mid (January) and late winter (March).

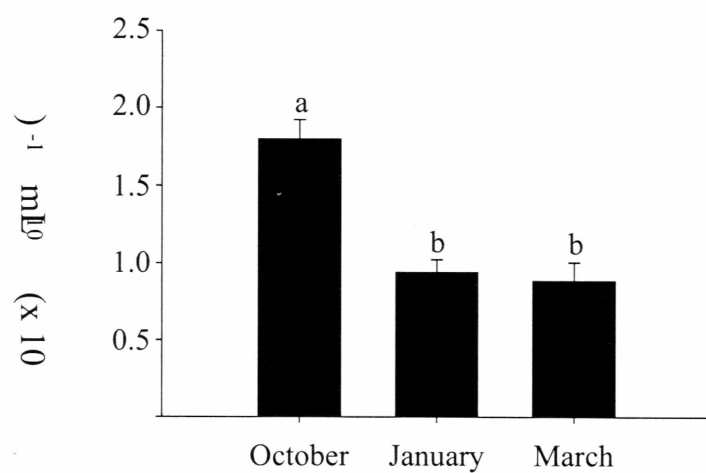


Figure 1.3. Bacterial counts ($\times 10^{10} \text{ mL}^{-1}$) in rumen fluid from muskoxen ($n = 4$) during winter. Different letters indicate significant differences between periods ($P < 0.05$).

CHAPTER 2: REGULATION OF FERMENTATION DURING SEASONAL FLUCTUATIONS IN FOOD INTAKE¹

ABSTRACT. — We studied rumen fermentation of castrated adult muskoxen (*Ovibos moschatus*) during periods of low (May) and high (August) food intake. Turnover time (17 ± 1.8 h) and volume (26 ± 3.9 L) of rumen fluid were consistent between May and August and among days within each season. Rumen osmolality (271.9 ± 16.4 vs. 245.9 ± 11.4 mOsmol.kg⁻¹) and pH (6.81 ± 0.31 vs. 6.39 ± 0.15) were higher in May than in August, indicating a shift in the allostatic set point. Rumen fluid pH was more variable in May than in August both before and after a single meal of fermentable substrate even though fermentation acids were lower in May than in August (101.0 ± 11.0 vs. 126.0 ± 8.74 mM). Changing proportions of minor fermentation acids indicated a shift in metabolic pathways even though bacterial numbers were similar between seasons ($6.4 \pm 5.8 \times 10^9$.mL⁻¹). Allostatic set points probably alter the homeostatic range of conditions and the microbial diversity of fermentation in herbivores in highly seasonal environments.

Keywords: arctic, bacteria, homeostasis, hyperphagia, rumen

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INTRODUCTION

Animals that rely on digestive fermentation for their principle source of energy must maintain stable conditions of temperature, osmolality, and acidity for their microbial symbionts (Hungate, 1966). Foregut fermentation systems such as the rumen are more vulnerable to fluctuations in the physical and chemical properties of food and water than fermentation systems in the lower tract (Stevens and Hume, 1995). Rumen microorganisms handle a wide variety of fermentative substrates from toxins to structural carbohydrates, proteins, and starches. Large imbalances in substrate load, such as excess starch, can force the rumen microbial ecosystem into disequilibrium resulting in excess production of gas, fermentation acids, and even death of the host (Russell and Rychlik, 2001; Owens *et al.*, 1998). The rumen is a complex of mutualistic relationships not only between the host and its microbes but also among the microbial phylotypes (Hungate, 1966; Stahl *et al.*, 1988). Digestive fermentation is, therefore, an outcome of multiple routes of metabolism with cascading pools of substrate and product. Rumen microbes are reliant upon the substrates consumed as food by the host. The host also mixes the substrates and the microbes, tritulates solid substrates, and maintains temperature, osmolality and acid balance that serve the microbial population (Russell, 2002). Natural shifts in food intake alter substrate availability to the microbes and impose a greater burden for services from the host. The rumen may be considered an allostatic system (Sterling and Eyer, 1988) where changes in food selection and intake by the animal shift conditions to new set points. Each meal may be considered a short-term load of substrate that tests the homeostatic response to acute shifts in rumen conditions.

Muskoxen are grazing ruminants (*Ovibos moschatus*) from highly seasonal arctic habitats where food abundance and composition changes widely throughout the year (Staal and Thing, 1991; Blix, 2005). Ruminal fermentation accounts for 80% of cellulose degradation in muskoxen (Barboza *et al.*, 2006). Substrate loads for rumen microbes can increase with food intakes of muskoxen by 74% from spring (hypophagia) to autumn (hyperphagia; Barboza *et al.*, 2006). Unstable or variable conditions within the rumen may be associated with reductions in the number and activity of bacteria in muskoxen during winter (Barboza *et al.*, 2006). Declines in rumen pH accompany seasonal hyperphagia as well as the consumption of fermentable supplements by muskoxen (Barboza *et al.*, 2006). We do not know whether rumen conditions of temperature, osmolality, and pH are more variable in response to seasonal changes in natural food intake or meal consumption in muskoxen.

Concentrations of short chain fatty acids (SCFA) produced from fermentation increase in concert with seasonal food intake in muskoxen (Barboza *et al.*, 2006). Concomitant changes in the pattern of SCFA concentration also suggest small shifts in substrate degradation pathways. We do not know if patterns of SCFA concentration change with meal consumption in the same fashion across seasons. Patterns of SCFA infer diversity of substrate and probably the expression of pathways by novel enzymes and/or novel microorganisms. We do not know whether a variable rumen environment is associated with seasonal diversity of microbial function or changes in substrate load.

We quantified the rumen microbial environment of muskoxen based on diurnal fluctuations in temperature, pH, osmolality, and SCFA concentration during May and

August. Food intakes are typically low (hypophagia) from January to June but increase (hyperphagia) from August through October as body mass is gained (Peltier *et al.*, 2003). We tested the effect of substrate load in each period by providing muskoxen with an acute load of fermentable material as part of their normal supplement of minerals. We compared two external markers (Cr-EDTA; Co-EDTA) to determine daily changes in rumen volume and fluid outflow. Numbers of rumen bacteria and concentrations of SCFA were measured during each season to assess the effect of substrate load on microbial dynamics.

MATERIALS AND METHODS

Animals. —

Captive muskoxen (*Ovibos moschatus*) were studied at the R. G. White Large Animal Research Station (Fairbanks, AK; 65N, 146W) under protocol #03-21 of the Institutional Animal Care and Use Committee, University of Alaska, Fairbanks. We used 4 castrated adult muskoxen (6.4 to 9.5 yrs) that had been surgically cannulated at the rumen 3 years prior to the experiment. Animals were housed in a large pen (2,043 m²) with water and hay (*Bromus* sp.) provided *ad libitum*. A pelleted mineral supplement (M-Ration, Alaska Pet and Garden, Anchorage, Alaska) was provided twice each week at 17.5 g·kg^{-0.75} body mass. The supplement consisted of starches and soluble proteins from cereal grains that are more rapidly degraded in the rumen than grass hay (Barboza *et al.*, 2006). Seasonal experiments were conducted during the typical periods of low (May) and high (August) food intakes (Peltier *et al.*, 2003). Losses in body mass of castrated

males during May accompany hypophagia whereas gains in body mass during August accompany hyperphagia (Peltier *et al.*, 2003). Body mass was routinely recorded (± 0.5 kg; model 703 scale, Tru-Test, San Antonio, Texas) during handling and averaged for each month from 2002-2005 to describe one full annual cycle.

Food and Digesta Composition. —

Common hay and individual supplement were collected periodically throughout the study to assess feed composition (Table 2.1). Samples were dried for 48 h at 50°C to determine dry matter content before being ground in a Wiley mill (Arthur Thompson Company, Philadelphia, Pennsylvania) with 2 mm mesh screen for further analyses. Feeds were dried to constant mass at 80°C to determine analytical dry matter. Ash content was measured by combusting 1 g of dried material for 8 h at 500°C in a muffle furnace (Barnstead International, Dubuque, Iowa). Fiber extractions followed the procedures of Peltier *et al.* (2003) and were performed sequentially as follows: neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin. Content of hemicellulose was determined as the difference between NDF and ADF whereas content of cellulose was determined as the difference between ADF and lignin (Van Soest *et al.*, 1991; Peltier *et al.*, 2003).

Fluid Markers. —

We used two solute markers (chromium and cobalt ethylene-diamine-tetraacetic acid, Co-EDTA and Cr-EDTA) to measure rumen volume and turnover time on two

subsequent days during May and August. Co-EDTA and Cr-EDTA were prepared according to Uden *et al.* (1980) as sodium salts. Markers were dosed in a cross-over design within each season to avoid confounding the effect of marker with dose day. Single doses of Co-EDTA (198 mg at $10\text{g}\cdot\text{L}^{-1}$ water) or Cr-EDTA (198 mg at $20\text{g}\cdot\text{L}^{-1}$ water) were injected into the rumen cannula 21 h after supplement on the first day. The second marker was dosed 24 h later to compare marker estimates of rumen volume and fluid turnover rates between days. Serial samples of rumen fluid were collected at 3, 6, 12, 24, 27, 30, 36, 48, 51, 57, and 78 h after dosing each marker. Rumen fluid samples were stored at -20°C for analysis.

A standard digestion cocktail composed of 70% v/v HNO_3 (1000 mL), 32 M H_2SO_4 (200 mL), 70% v/v HClO_4 (343 mL), and deionized water (57 mL) was used for analysis of minerals (Trumble *et al.*, 2003). We loaded flasks with 10 mL rumen fluid and 12 mL digestion cocktail and digested in sequence from 65.5°C to 260°C for 4 h to completely remove any organic material. Digests were assayed by directly coupled plasma spectrometry (Iris DCP, Thermo Elemental, Cheshire, UK) against certified standards.

Rumen Environmental Parameters. —

Rumen temperatures were recorded at an accuracy of 0.03°C every 30 s for 5 days in each study period (HOBO Water Temp Pro Data Loggers, Onset Computer Corp., Bourne, Massachusetts). Temperature probes were tethered to each rumen cannula with a

swivel attached to 45 cm of rope, allowing for full rotational motion (Crater and Barboza, unpublished data).

Diurnal variations in rumen conditions were assessed by sampling fluid for 3 consecutive days at 06:00, 12:00, and 18:00 (Alaska Standard Time). Supplement was withheld for 24 h before beginning the diurnal series of samples.. Rumen fluid samples were also obtained 1 h prior to supplement and 3, 6, 9, 21, and 24 h post supplement to assess the effects of fermentable substrate load on the rumen microbial ecosystem. Approximately 300 mL of rumen digesta was removed from the top of the rumen raft with forceps and placed into insulated flasks. We squeezed each sample through a Brazil Bodum French press (1543-01US, Bodum, Wisconsin, USA) in a 250 mL beaker to obtain liquid samples for measurements of pH, osmolality, SCFA, and bacterial numbers.

We measured diurnal variations in rumen pH (± 0.01 units; Automatic Temperature Compensation Electrode; Corning Electrodes) within 5 minutes of extraction from the rumen. Osmolality was measured in freshly collected samples at room temperature (18°C) by vapor pressure (Wescor Vapro 5520, Logan, Utah) throughout each season.

Rumen Bacteria and SCFA Concentrations. —

Rumen fluid was preserved for bacterial counts by collecting 15 mL aliquots into an equal volume of 10% formalin. Bacteria were counted with a phase contrast microscope (Olympus Optical Co., Japan) using a hemocytometer chamber (Bright Line

Counting Chamber, Hausser Scientific, Horsham, PA). All formalin samples were diluted to provide 40 to 70 bacteria per cell.

SCFA were prepared according to Barboza *et al.* (2006) by adding 0.7 g of 25% w/v metaphosphoric acid to 3.3 g of rumen fluid and centrifuging at 5000 x g (Sorvall Instruments RC5C, Dupont Company, Newtown, CT) for 10 min at 2°C. We used gas chromatography (HP model 5890, Agilent Technologies, Wilmington, DE) to analyze rumen fluid SCFA. Crotonic acid was used as the internal standard to measure acetate, propionate, n-butyrate, iso-butyrate, valerate, iso-valerate and caproate against the retention times of certified standards. We analyzed elution profiles with HP 3365 Series I Chemstation (Agilent Technologies, Wilmington, DE). All SCFA concentrations were expressed on the basis of whole rumen fluid (mM) after correcting for dilutions with mercuric chloride and metaphosphoric acid.

Calculations. —

Turnover time and pool size were calculated from the concentration of Co or Cr in sequential samples of rumen fluid. We assumed that each marker was instantly mixed with aqueous fluids and that the decline in marker concentration followed first order kinetics. The natural logarithm of the decline in marker concentration was fitted to a least-squares regression against time since dosing. Turnover time (h) was calculated as the negative inverse slope of the regression ($-h^{-1}$) while the size of rumen fluid pool was calculated by dividing the dose (μg Co or Cr) with the concentration of marker ($\mu\text{g}\cdot\text{gDM}^{-1}$) predicted at time zero from regression (Barboza *et al.*, 2006).

Statistical analyses were performed in Systat 10.2 (SPSS Inc., Chicago, Illinois) to test the main effect of season. We used a general linear model with repeated measures to test the effect of dosing order for fluid markers within animals. General linear models of body mass, rumen temperature, rumen fluid pH, osmolality, total SCFA concentrations, and bacterial counts were compared with season and time from supplement as factors within animals. We averaged rumen temperatures that were recorded in 30 s intervals, over 10 min intervals (5 min before and 5 min after rumen sampling time) for comparison with other rumen parameters. We used repeated measures of SCFA concentration as well as the proportional concentration of each acid to compare seasons and time from supplement. Proportional concentrations were transformed to square root of the arcsine to meet assumptions of normality for ANOVA (Zar, 1999). Variation in rumen conditions within animals (Δ) was calculated as the difference between sequential measures of marker kinetics, range (maximum – minimum) of diurnal measures (temperature, osmolality, and pH) and the maximum absolute change in conditions before and after supplementation. Statistical significance was determined at $\alpha < 0.05$.

RESULTS

Muskoxen followed the predicted pattern of mass loss through May, and recovery of body mass through September (Fig. 2.1) that has been previously associated with hypophagia (mass loss) and hyperphagia (mass gain; Peltier et al. 2003). Absolute changes in body mass varied between animals and between years. Mass changes were

smaller in 2004 than in 2003 even though all animals were mature (> 5 years) and no longer growing in both years. All animals lost mass through May and into June in 2004 ($P < 0.05$). Autumn regain of body mass was smaller in 2004 than in 2003. Three animals gained mass from July to August 2004 whereas the remaining animal maintained mass from July to August 2004.

Fluid Markers. —

Marker kinetics were similar between Cr-EDTA and Co-EDTA and not affected by the order in which doses were administered ($P = 0.65$). Variation within animals (Δ) in pool size and turnover time was similar between May and August ($P > 0.05$; Table 2); that is, variability in marker kinetics did not change with season. The volume and turnover time for rumen fluid was similar between May (22.9 ± 12.4 L; 17.5 ± 3.5 h) and August (26.0 ± 4.5 L; 16.4 ± 6.3 h; Table 2.2). Consequently, concentrations of metabolites or microbes in the rumen were not affected by seasonal changes in rumen volume or fluid outflow.

Diurnal Measurements. —

Diurnal variations in rumen conditions were small within each season when supplement was withheld. Rumen temperature of muskoxen did not vary significantly through the day ($38.8 \pm 0.29^\circ\text{C}$; Fig. 2.2) or between seasons. Neither rumen pH nor osmolality changed during the day in either May or August. Rumen conditions in August were more dilute (245.9 ± 11.4 vs. 271.9 ± 16.4 mOsmol.kg⁻¹) and acidic (6.39 ± 0.15 vs.

6.81 ± 0.31) than in May (Fig. 2.2). Although rumen pH was more variable within animals (Δ) in May than in August (Fig. 2.2 E, F), diurnal differences between maxima and minima were similar between seasons for temperature and osmolality.

Supplement. —

Average rumen temperatures were higher after supplementation in August ($39.2 \pm 0.40^\circ\text{C}$) than in May ($38.7 \pm 0.44^\circ\text{C}$; Fig. 2.3). Supplementation increased rumen osmolality within 3 h in both seasons (Fig 2.3). Rumen fluid osmolality returned to pre-supplement levels more quickly in August than in May (21 vs. 24 h). Supplementation progressively reduced rumen pH for up to 6 h but pH values returned to pre-supplement levels after 24 h in both seasons (Fig. 2.3). The maximum depression in rumen pH after supplementation (Δ) was greater in May than in August (Fig. 2.3).

Rumen Bacteria and SCFA Concentrations. —

Concentrations of bacteria in rumen fluid did not change between seasons or with time from supplement (Fig. 2.4) even though fermentation acids varied with season (Fig. 2.5). Bacterial numbers were, however, more variable (Δ) in May than in August (Fig. 2.4). Fermentation acids were more concentrated during hyperphagia in August (126.0 ± 8.74 mM) than during hypophagia in May (101.0 ± 11.0 mM; Fig. 2.5) both before and after supplementation. Supplementation increased total SCFA concentrations for up to 9 h in both seasons. Molar proportions of major SCFA were higher in August than in May for acetate (75 ± 2.0 vs. $72 \pm 1.9\%$) and n-butyrate (6.6 ± 0.73 vs. $5.7 \pm 0.34\%$) but lower

for propionate (16 ± 1.3 vs. 21 ± 2.1 %). Supplementation did not affect the proportions of major SCFA within season but did depress minor SCFA for up to 9 h (Fig. 2.5).

Average proportions and concentrations of minor acids were greater in August than in May especially for valerate (0.67 ± 0.14 vs. $0.52 \pm 0.08\%$) and caproate (0.20 ± 0.04 vs. $0.09 \pm 0.003\%$) .

DISCUSSION

Castrated muskoxen lost body mass from April to July 2004 but began to recover mass as food intake increased from August to October 2004 (Peltier *et al.*, 2003). Our study measured rumen conditions when animals were lowest in mass (May) and regaining mass (August) during the typical periods of hypophagia and hyperphagia. Although absolute changes in body mass may vary between years, seasonal food intakes in muskoxen and ruminants at high latitudes follow a predictable pattern cued by photoperiod (Rhind *et al.*, 2002; White *et al.*, 1987). Consistent measures of rumen volume reflected similar body mass between May and August (2004) on the declining and rising phase of the annual cycle (Table 2.2; Fig. 2.1). Changes in rumen temperature and solute concentration between May and August were, therefore, not confounded by shifts in rumen dynamics or food composition (Table 2.1). Consequently, changes in rumen conditions between May and August were probably related to the seasonal pattern of food intake and the endogenous response to feeding in muskoxen (Adamczewski *et al.*, 1994; Lawler and White, 2003; Peltier *et al.*, 2003). Castrated muskoxen can increase food intake by 74% between spring (May-June) and autumn (August-September) by increasing gut fill

(31-34%) and microbial degradation (100%) which conserve retention time (10%) and, thus, the time available for fermentation between seasons (Barboza *et al.*, 2006).

Because rumen dynamics were conserved between May and August in this study, changes in the temperature and the concentration of solutes, fermentation acids, and bacteria in rumen fluid probably reflect the secretions of the host and the activities of the microbes.

Changes in food intake and mucosal exchange between seasons alter the allostatic set points around which rumen pH and osmolality are regulated. Increases in the concentration of SCFA between May and August (Fig. 2.5) were consistent with lower average pH (Fig. 2.2) and with greater microbial activity that would accompany seasonal changes in substrate load. Conversely, rumen osmolality was lower in August than in May even though substrate loads were probably increased by food intake. Seasonal reductions in osmolality (Fig. 2.2; 2.3) may be associated with increases in water flux during hyperphagia (Crater and Barboza, unpublished data; White *et al.*, 1984) as well as increases in mucosal flux that dilute and remove solutes from digesta soon after a meal (Russell, 1988). Large seasonal changes in the loads of solute such as SCFA may be offset by a high capacity for absorption in arctic ruminants throughout the year. Ruminal uptake of n-butyrate *in vitro* does not change with season despite changes in food intake of reindeer (Storeheier *et al.*, 2003). This suggestion requires further confirmation with direct measures of mucosal exchange between digesta and blood of northern ungulates *in vivo*.

The daily production of SCFA in the rumen can be estimated from the digestible energy intakes of castrated muskoxen in spring (May-June; $554 \text{ kJ.kg}^{-0.75}.\text{d}^{-1}$) and autumn (August-September; $923 \text{ kJ.kg}^{-0.75}.\text{d}^{-1}$; Peltier *et al.*, 2003). If we assume that 79% of the total digestible energy of the diet is released within the rumen (Barboza *et al.*, 2006), then 31 MJ.d^{-1} and 51 MJ.d^{-1} of fermentation acids are released during hypophagia and hyperphagia, respectively. Molar proportions of SCFA (Fig. 2.5) can be combined with calorific values for each acid (Blaxter, 1962) to subsequently estimate a daily load of 31 mol.d^{-1} and 50 mol.d^{-1} of total SCFA during May and August, respectively. An increase in acid load by 62% during August must be supported by larger secretions of buffering ions and greater uptake by the rumen mucosa when passage rates are unchanged. This suggestion is supported by a low incidence of acidosis and bloat in captive muskoxen even though these animals produce SCFA at rates similar to domestic ruminants fed fermentable substrates (55 mol.d^{-1}) for fattening or milk production (Owens *et al.*, 1997).

Consistent measures of rumen volume and fluid turnover rates indicate that fluctuations in food intake between days are small within season (Table 2.2). Furthermore, rumen conditions did not vary during the day for temperature, osmolality, or pH (Fig. 2.2). Homeostasis of rumen conditions is favored by regular forage consumption and rumination that introduce and mix substrates and solutes for the microbial population throughout the day. Homeostatic control in muskoxen is typical of domestic ruminants that follow a similar time course of rumen pH and osmolality following an acute load of fermentable substrate (Fig. 2.3; Dehority, 2003, Van Soest, 1994). Rumen conditions of pH, osmolality, and temperature are not independent of each

other. Fermentable substrates increased the concentration of SCFA in both May and August (Fig. 2.5) with a concomitant rise in the average temperature of the rumen. Microbial activities probably contribute to seasonal changes in the heat increment of feeding in muskoxen that result in higher post-prandial energy expenditures in summer than in winter (Lawler and White, 1997). Fermentations may be partly regulated by rumen temperature in muskoxen because maximum temperatures never exceed 41°C but can drop to 26°C for several hours (Crater and Barboza, unpublished data). Heat of fermentation may, therefore, set an upper limit for microbial degradation during hyperphagia. Greater variability in rumen pH during May both with and without supplementation (Fig. 2.2; 2.3) and slower recovery of rumen osmolality after supplementation in May indicate less capacity to buffer the rumen or remove acids even though food intake and SCFA production are probably low. Consequently, homeostatic control of pH is lower in May than in August. Buffering capacity of the rumen is partly dependent upon bicarbonate secretion across the mucosa and in saliva (Kay, 1960) that may be altered by both exogenous (*e.g.*, chemical properties of the feed) and endogenous (*e.g.*, stimulation of the vagus nerve) factors (Russell, 2002). Secretions of ions may vary with both season and substrate as food intakes increase and as plants grow and senesce.

Allostatic setpoints and homeostatic ranges of rumen conditions can select for different phylotypes of bacteria with varying substrate affinities and metabolic pathways (Van Gylswyk, 1977; Wallace, 1978; Wolin, 1964). Bacteria that degrade cellulose are inhibited at pH less than 6.0 (Mould *et al.*, 1983-84; Russell and Dombrowski, 1980; Russell, 1991; Russell, 1992) when acid-resistant phylotypes predominate. Greater

variability in bacterial concentrations during May may reflect the combined effect of low food intake (Crater and Barboza, unpublished data) and a wider range of pH than in August. Concentrations of rumen bacteria may not, however, reflect the genetic and metabolic diversity of the bacterial assemblage. Seasonal changes in minor fermentation acids (Fig. 2.5) that accompany changes in food intake could be associated with changes in the microbial population and shifts in the optima for rumen conditions and substrate availability. We offer two explanations for potential shifts in the composition of rumen bacteria between hypophagia and hyperphagia. High degradation rates may be attained by a complementary bacterial assemblage with broad substrate utilization or a narrow range of phylotypes that utilize the most abundant substrates in the diet (Fig. 2.6 A). Seasonal emergence of novel microorganisms may be discerned from changes in common 16s rDNA sequences (Fig. 2.6 B; Woese, 2002). If core phylotypes change in relative abundance, then the type of 16s rDNA sequences will not vary with season but will change in relative number. Shifts in microbial population structure may be further assessed by diversity of genes and expression within phylotypes across seasons (Fig. 2.6 B). Shifts in microbial diversity have been reported for ruminants following changes in diet (Stahl *et al.*, 1988; Tajima *et al.*, 2001) but have not been related to natural changes in food intake that accompany seasonal cycles. Changes in food intake drive allostatic set points that may further influence the homeostatic range as diet and substrate diversity change with season. Food abundance and quality may, therefore, affect the microbial assemblages that underlie energy and protein supply to wild ruminants, especially in highly seasonal environments.

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Table 2.1. Mean composition of dry matter in hay and supplement fed to cannulated muskoxen during May (hypophagia) and August (hyperphagia). Variation (CV) within samples was less than 5%.

Component (g·DMg ⁻¹)	Hay		Supplement	
	May	August	May	August
Dry Matter	0.96	0.96	0.98	0.97
NDF	0.62	0.67	0.30	0.33
Hemicellulose	0.30	0.31	0.15	0.18
ADF	0.32	0.35	0.15	0.15
Cellulose	0.29	0.32	0.13	0.13
Lignin	0.03	0.03	0.02	0.02
Ash	0.04	0.07	0.08	0.09

Table 2.2. Repeated measures (mean \pm SD) of body mass, rumen pool size (L) and turnover time (h) for fluid markers (Co-EDTA and Cr-EDTA) in muskoxen (n = 4) during May (hypohagia) and August (hyperphagia) with the difference between sequential measures within animals (Δ). Parameters were not significantly different between markers or between seasons ($P > 0.05$).

Parameter	May – Hypohagia			August - Hyperphagia		
	Co	Cr	Δ	Co	Cr	Δ
Body Mass (kg)	289 \pm 14	291 \pm 13	4.5 \pm 3.4	289 \pm 20	287 \pm 18	3.3 \pm 4.6
Total Turnover (h)	19 \pm 3	16 \pm 3	2.3 \pm 2.1	15 \pm 7	18 \pm 7	4.5 \pm 2.3
Pool (L)	24 \pm 5	22 \pm 4	4.1 \pm 3.1	30 \pm 9	29 \pm 9	8.6 \pm 7.3

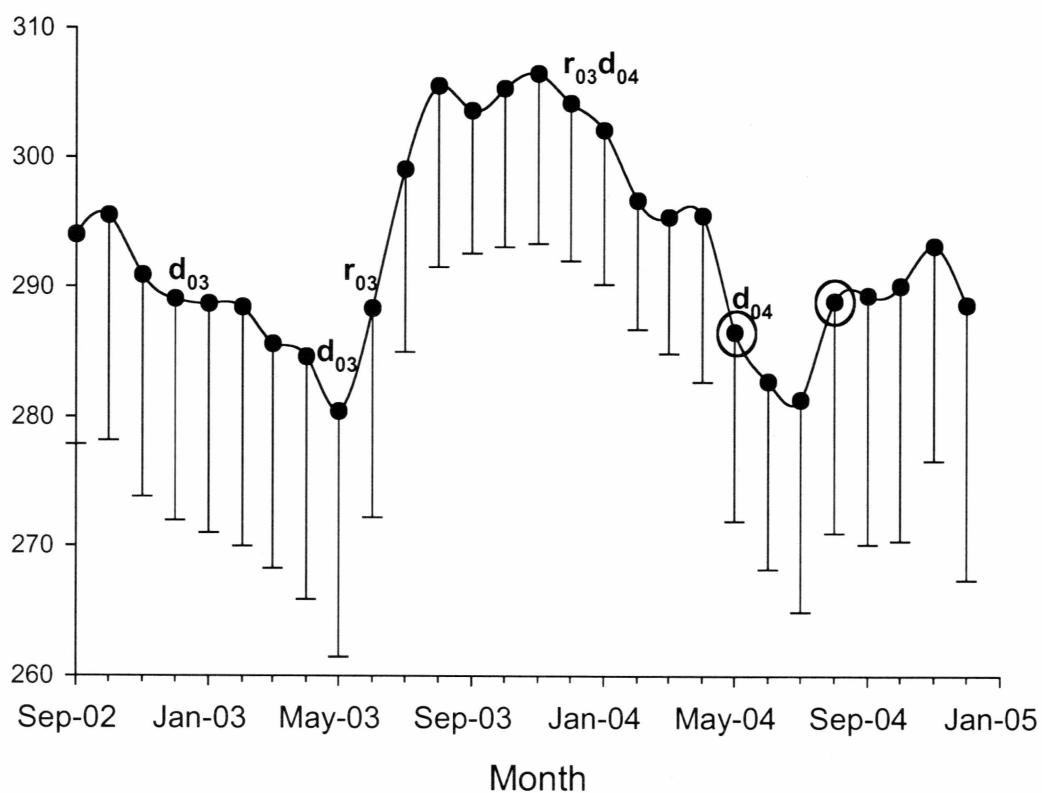


Figure 2.1. Body mass (kg) of castrated muskoxen ($n = 4$; mean - SD) fed grass hay *ad libitum* from 2002 through 2004. Circles correspond to study periods in May and August 2004. Letters indicate significant differences in body mass ($P < 0.05$) associated with seasonal declines (d) and rises (r) in 2003 or 2004.

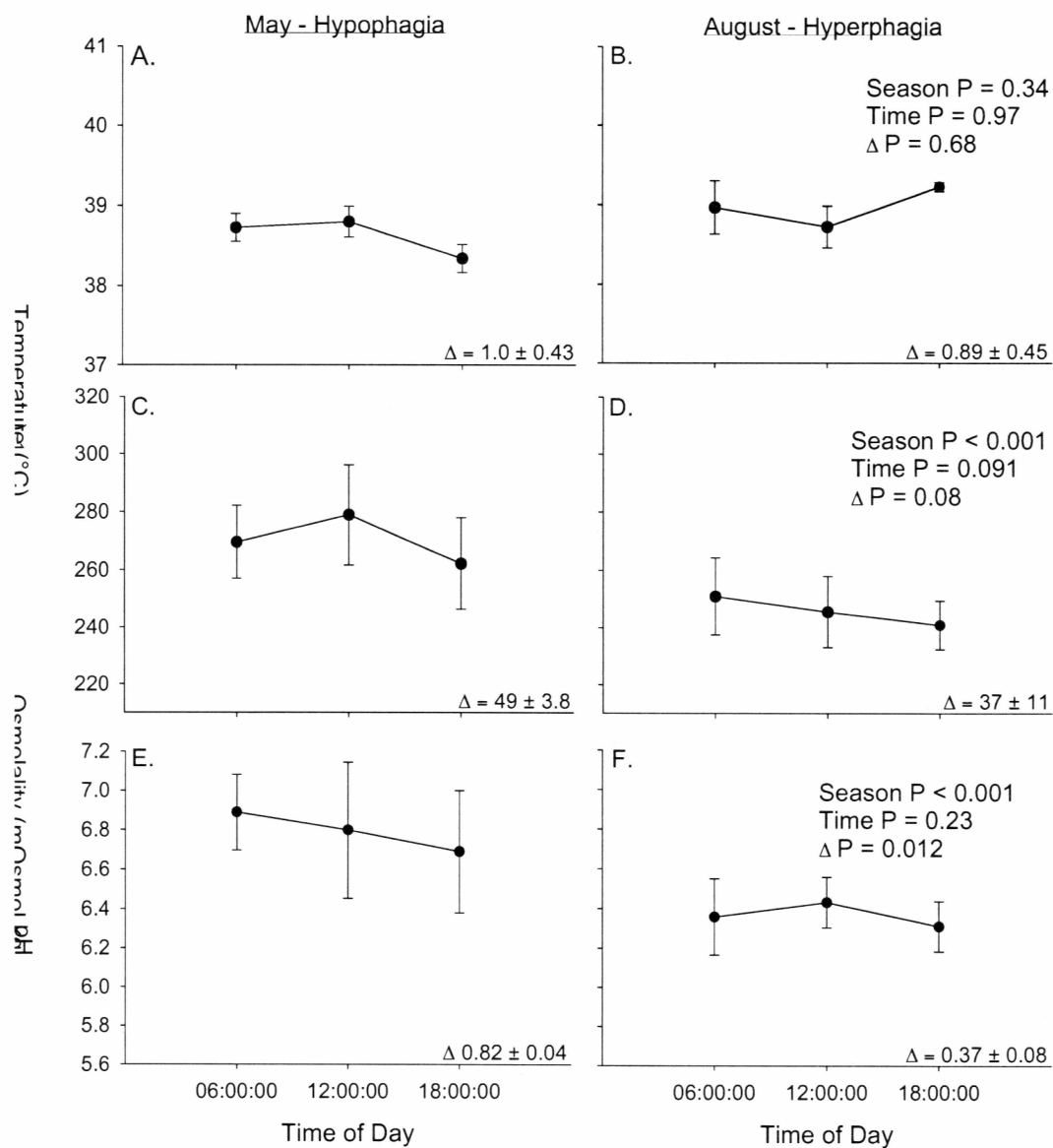


Figure 2.2. Diurnal variation in rumen temperature (°C; A, B), rumen fluid osmolality (mOsmol.kg⁻¹; C, D), and pH (E, F) in muskoxen (n=4; mean ± SD). Range (Δ) is the difference between maximum and minimum values within each animal.

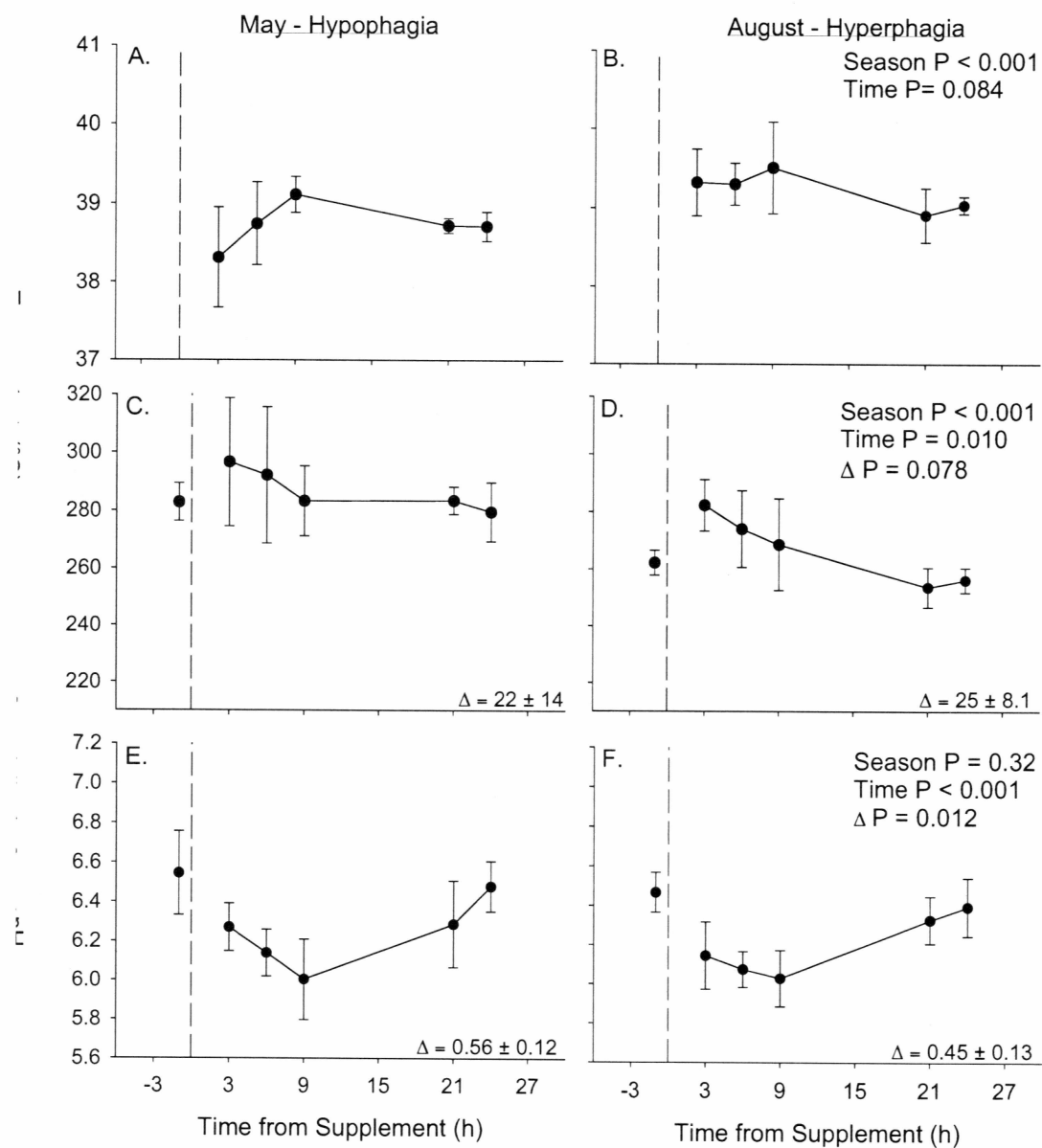


Figure 2.3. Effect of supplementation on rumen temperature ($^{\circ}\text{C}$; A, B), rumen fluid osmolality (mOsmol.kg^{-1} ; C, D), and pH (E, F) in muskoxen ($n=4$; mean \pm SD). Vertical dashed line indicates the time at which fermentable supplement was provided. Maximum effect (Δ) was the largest absolute difference between values before and after supplementation within each animal.

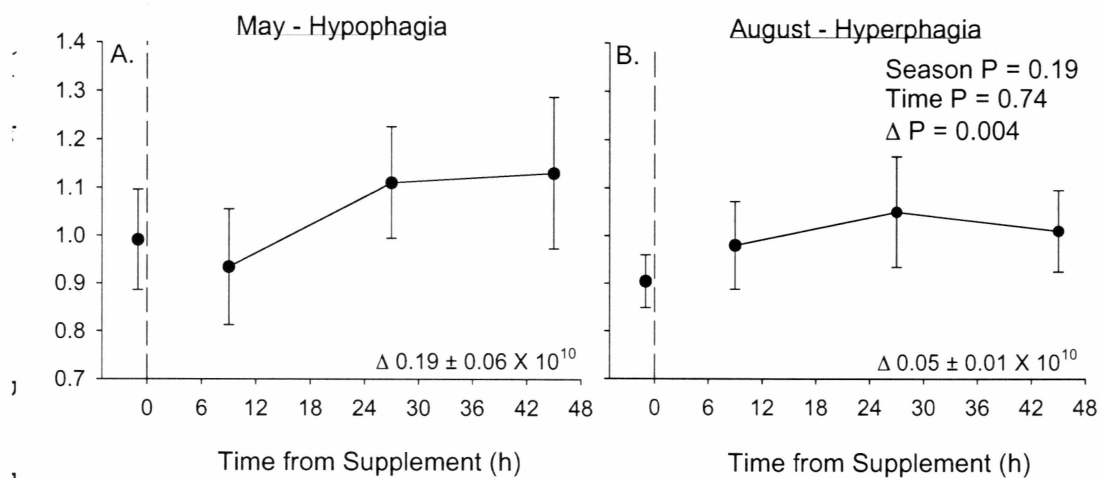


Figure 2.4. Bacterial counts (x 10¹⁰ mL⁻¹) in rumen fluid from muskoxen (n = 4; mean ± SD) during hypophagia (May; A) and hyperphagia (August; B). Vertical dashed line indicates the time at which fermentable supplement was provided. Maximum effect (Δ) was the largest absolute difference between values before and after supplementation within each animal.

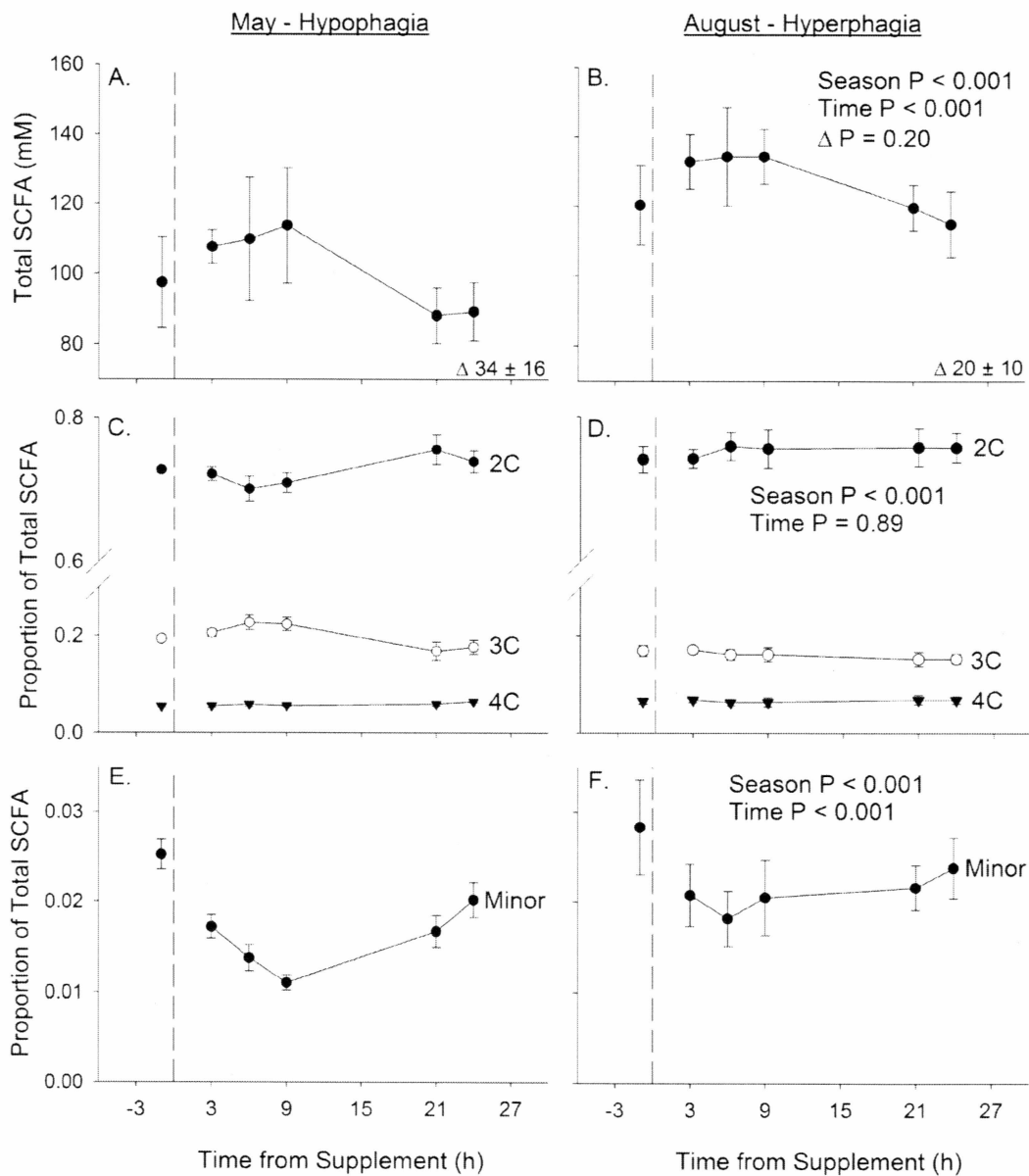


Figure 2.5. Effect of supplementation on total concentration of SCFA (mM; A, B) and the molar proportion (mM \div total mM) of major (C, D) and minor (E, F) acids in the rumen of muskoxen. Major acids were acetate (2C), propionate (3C) and n-butyrate (4C). Minor acids were the sum of valerate, isovalerate, isobutyrate, and caproate. Maximum effect (Δ) was the largest absolute difference between total SCFA concentrations before and after supplementation within each animal.

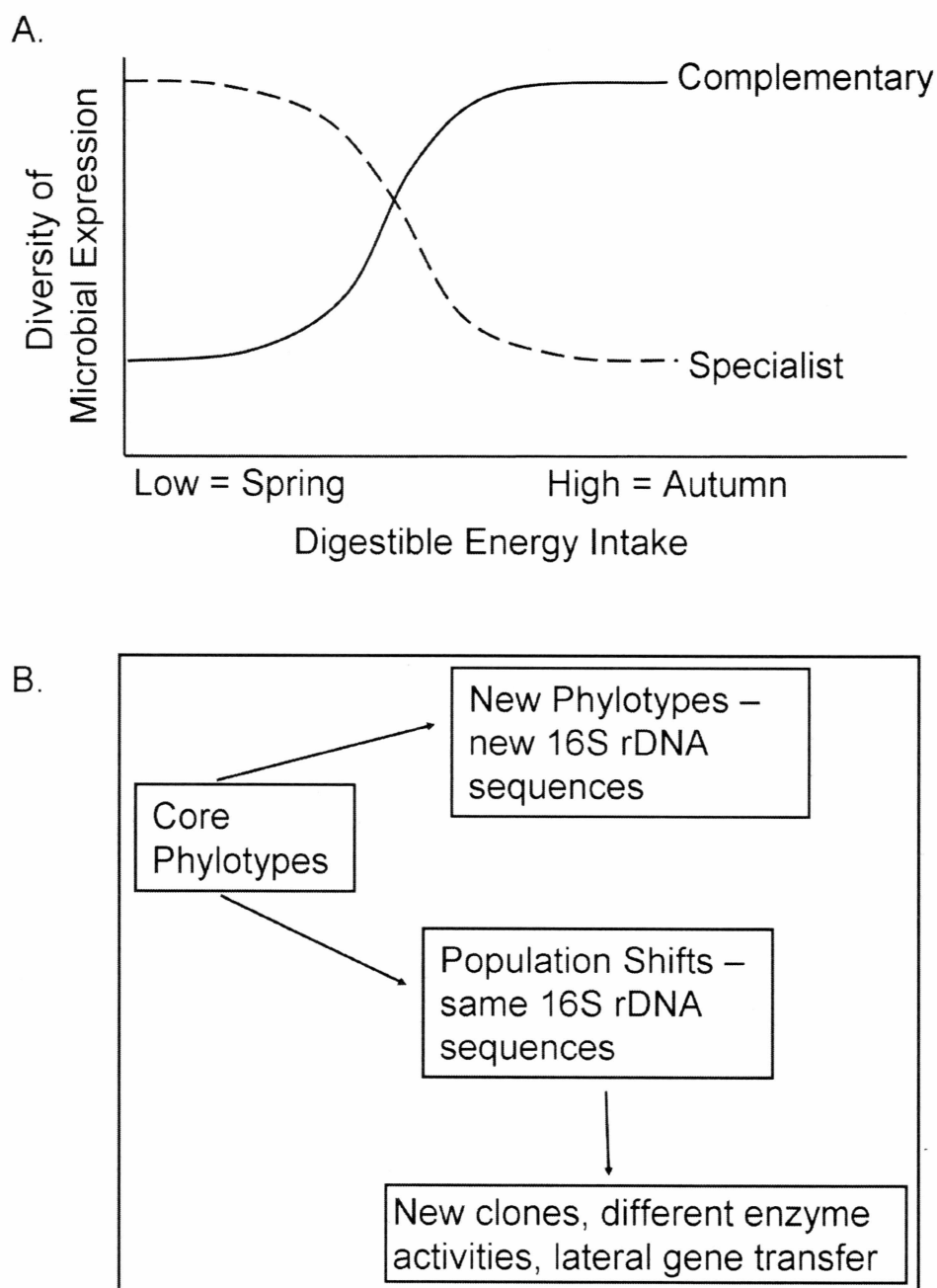


Figure 2.6. Schematic model of shifts in microbial diversity of the rumen during seasonal changes in food intake. A. Hypothetical responses of diversity to substrate supply in food. B. Methods of discriminating between complementary and specialist assemblages.

CHAPTER 3. ISOLATION OF BACTERIAL 16S RDNA FROM DIGESTA IN DOMESTIC AND WILD RUMINANTS¹

ABSTRACT. — Molecular approaches to evaluating bacterial diversity in the digestive tract are often limited by extractions of low quality DNA from heterogeneous samples of digesta. Current techniques use either a quick method that has a low DNA yield or a higher yielding and time-consuming method that utilizes bead-beating and phenol/chloroform extraction. We describe a method to separate liquid and particulate digesta phases and to obtain high quality DNA for downstream enzymatic reactions. The DNA extraction protocol follows a modification to the existing QIAamp DNA Stool Mini Kit. Our modified procedure provides 63% more bacterial DNA for PCR-amplification and DNA sequencing.

Keywords: rumen bacteria, DNA extraction, digesta phases

¹Prepared for submission in *BioTechniques* as Crater, A. R., R. J. Forster and P. S.

Barboza. Isolation of bacterial 16s rDNA from liquid and particulate phases of digesta in domestic and wild ruminants.

INTRODUCTION

Rumen microorganisms dictate the use of substrates in wild and captive ruminants by the fermentation pathways they employ. Although rumen bacteria typically use energy substrates in food such as carbohydrates, proteins, and lipids, microbial fermentations may also detoxify plant secondary metabolites and nitrogenous wastes. Therefore, rumen microbes influence metabolic and immunological aspects of the host animal's physiology (1). Estimates of microbial diversity are important in determining the nutritional niche of the ruminant. Moreover, novel phylotypes of rumen bacteria may harbor novel genes that enhance cellulolytic degradation within the foregut and increase the fermentation end-products available to the host.

Researchers have traditionally utilized culture-based techniques that tend to underestimate diversity in samples from the rumen (2) because rumen microbes require meticulous anaerobic conditions. Molecular methods, based on sequencing 16S rDNA, provide a more robust assessment of microbial populations. The variable regions of 16S rRNA genes contain sequences that may be unique to genus, species or strains of bacteria (3) and permit differentiation of phylotypes. Molecular approaches that measure rumen bacterial diversity have been limited by the ability to obtain representative PCR-quality DNA from digesta samples. Extractive procedures can be physical (*e.g.*, bead beating), chemical (*e.g.*, Sodium-Dodecyl-Sulfate and phenolchloroform) or enzymatic (*e.g.*, proteinase K). Single approaches tend to extract low quality DNA from rumen bacteria (4, 5) whereas combined approaches can improve both the quality and quantity of DNA

(6). However, combined approaches are time consuming and may not suffice for further molecular processing of bacteria from heterogeneous samples such as digesta (7).

Bacteria are associated with both the liquid and particulate fractions of rumen digesta. Liquid and particulate associated bacteria differ in abundance and in their use of substrates. Quantification of bacterial phylotypes in the rumen is contingent upon equal extraction of DNA from both digesta phases. Liquid associated bacteria are more accessible and easier to lyse than bacteria associated with the particulate fraction. Consequently, the bulk of rumen bacteria associated with the particulate fraction are understudied. We describe a method for separating rumen bacteria from both liquid and particulate fractions with subsequent extraction of DNA from both fractions.

MATERIALS AND METHODS

Separation of Liquid and Particulate Associated Rumen Bacteria. —

Rumen digesta was collected from dairy cattle (*Bos taurus*) and muskoxen (*Ovibos moschatus*) fed grass hay. Digesta was sampled through a rumen cannula from the raft of long fibers in the dorsal rumen. Samples of digesta were collected into insulated bottles to maintain temperature and transferred to an anaerobic glove bag (129.5 X 147.3 cm; Aldrich Two- hand AtmosBag, cat. no. Z10,608-9). The AtmosBag was evacuated and purged 3 times with anaerobic CO₂ before filling (Praxair UN 1013)

A Bodum Press (#1543-01US, Bodum, Wisconsin, USA) was used to extrude liquids from 100 mL of whole digesta. Liquid samples were removed in 4 aliquots of 1 mL each and dispensed into 2 mL screw top vials (Nalgene, Rochester, NY, USA).

Particulate digesta were rinsed with phosphate rinsing buffer and methylcellulose releasing buffer. Phosphate rinsing buffer (PRB) consisted of 5.23 g K_2HPO_4 , 2.72 g KH_2PO_4 , and 3.0 g $NaHCO_4$ in 1 L, with 0.5 ml of 0.1% resazurin as an oxygen indicator. The solution was degassed with anaerobic CO_2 . The PRB solution was transferred to an anaerobic glove bag before adding 20 mL of 2.5% w/v cysteine as a reducing agent. Methylcellulose releasing buffer was prepared with 1L of the complete PRB and 20 g methylcellulose. Methylcellulose was mixed into the buffer at room temperature and stored at 4°C to cool the solution and allow the methylcellulose to gel. The cooled solution was stirred to dissolve the methylcellulose before allowing the final solution to equilibrate to room temperature before use.

Particulate digesta that remained after decanting the initial rumen liquor was mixed with 100 mL of PRB before extruding the fluid through the Bodum Press. Extruded rinses, containing loosely associated bacteria from the particles, were discarded. Three rinses, mixes, and extrusions were completed before adding 100 mL of methylcellulose buffer to the remaining fibrous digesta. Digesta were briefly homogenized in the releasing buffer without heating the contents (Biohomogenizer, Biospec Products Inc., Bartlesville, OK, USA; 3 X 20 s bursts with 10 s between each burst). Bacteria that were released from the particles into the fluid phase were extruded with a Bodum Press to collect 4 X 5 mL samples into 14 mL Polypropylene Round-Bottom Falcon Tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The samples were centrifuged for 10 min at 10,000 X g (Sorvall RC-5C, Du Pont Company, Newton, CT, USA) and the supernatant was discarded. The remaining

bacterial pellet was resuspended in 1.4 mL ASL Buffer (Qiagen, Mississauga, ON, Canada) using a vortex mixer. Samples representing the particulate-attached bacteria were transferred to 2 mL Eppendorf tubes and stored at -80°C until further analyses.

Cell Lysis and DNA Extraction. —

Samples preserved in ASL buffer were heated in a waterbath at 95°C for 5 min and centrifuged at $20,000 \times g$ for 5 min to remove cellular fragments. The supernatant was separated and stored for DNA isolation using the QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada). Centrifuged pellets were resuspended in 1.4 mL of 0.4 M potassium phosphate buffer with a sonicator and vortex mixer. A series of enzymes was used to release nucleic acids from the solubilized pellet. The solubilized pellet was incubated for 1 h at 37°C with 100 μL of 100 $\text{mg}\cdot\text{mL}^{-1}$ lysozyme, 10 μL of 2.5 $\text{U}\cdot\mu\text{L}^{-1}$ mutanolysin, and 20 μL of 20 $\text{mg}\cdot\text{mL}^{-1}$ proteinase K. A bead beater (B. Braun Biotech, Allentown, PA) was used to break open recalcitrant cells and release DNA by heat sealing the sample with 0.2 g of 425-600 μm glass beads and 0.3 g of 710-1180 μm glass beads and beating for 3 min. Samples were centrifuged for 1 min at full speed ($16,000 \times g$) before proceeding with the protocol from the QIAamp® DNA Stool Mini Kit Handbook (step 5; page 16; Qiagen, Mississauga, ON, Canada). After cell lysis, the QIAamp® procedure used InhibitEX to eliminate DNA-damaging substances and PCR inhibitors from the samples, before purifying the DNA for PCR. Proteins were digested with Proteinase K and DNA was bound to the QIAamp® silica-gel membrane to wash away impurities and elute DNA from the spin column. The DNA produced from the

initial supernatant isolation and the DNA obtained from the subsequent bead-beating method were combined before proceeding to amplification of the 16S rRNA genes

Amplification of 16s rDNA by PCR. —

PCR amplifications were performed in 0.5 mL GeneAmp® Thin-Walled Reaction Tubes (Applied Biosystems, Foster City, CA). Each reaction mixture (200 µL; Table 3.1) consisted of 80 µL of 5X PCR buffer (final concentration 50 mM Tris, pH 8.3, 2.5 mM Invitrogen MgCl₂, 500 µg.mL⁻¹ Biolabs BSA), 10 µL of each dNTP (Sigma, D-7295 Lot No. 60K7029), 16 µL of the forward primer 27f OFRG (5' – AGRRTTTGATYHTGGYTCAG- 3') 16 µL of the reverse primer 1492 rOFRG (5' – GBTACCTTGTTACGACTT- 3'), 4 µL Amplitaq Gold DNA Polymerase (Applied Biosystems, Lot No. E13078, Foster City, CA), 70 µL H₂O, and 4 µL of the bacterial DNA sample. Reaction tubes were mixed by vortex and centrifuged at 1,500 X g for 3 min (Sorvall RT7, DuPont) before placing in a Bio-Rad iCycler thermal cycler (Mississauga, ON, Canada). Twenty amplification cycles were run as follows: 94°C for 10 min; 25 X (94°C, 30 sec; 48°C, 30 sec; 72°C, 4 min); 72°C for 4 min. Amplified samples were stored at 4°C.

To remove PCR primers, dNTPs, and enzymes from the DNA product, a Strataprep PCR Purification kit (Stratagene Cloning Protocol, PCR-Script™ Amp Cloning Kit, Cat. No. 400771, Lot No. 1190214) was used with 100 µL aliquots of the PCR reaction. We added 100 µL of DNA-binding solution to each aliquot, mixed, and transferred the solution to a microspin cup nested in a 2 mL tube. The samples were then

spun at full speed in a microcentrifuge for 30 sec and the eluant was discarded in the collection tube. We added 750 μL of 1 X Wash Buffer to the microspin cup, spun the samples at full speed for another 30 sec, and discarded the eluant. The samples were centrifuged once more to remove any residual 1 X Wash Buffer before transferring to a 1.5 mL Eppendorf tube. We added 50 μL of 10 mM Tris (pH 8.5) to the surface of the microspin cup fiber matrix and incubated at room temperature for 5 min. The sample was spun at full speed (16,000 X g) for 30 sec in a microcentrifuge to elute dsDNA from the fiber matrix.

Fluorometric measurements to quantify PCR products were made with a BioRad VersaFluorTM Fluorometer (BioRad, Richmond, CA). Standards consisted of 100 μL of 1000 ng.mL^{-1} cow thymase DNA plus 100 μL of 1/200 PicoGreen dsDNA quantitation reagent (Molecular probes, P-7581, Lot No. 3161-3) while blanks consisted of 100 μL TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) plus 100 μL PicoGreen. Each rumen bacterial sample consisted of 10 μL (1:10 dilution) in 100 μL of TE buffer.

The PCR product was also verified by gel electrophoresis (Microprocessor Controlled Electrophoresis EC703, E-C Apparatus Corporation, FA). A 10% agarose gel containing ethidium bromide (5 mg.mL^{-1}) was loaded with 10 μL of 1 kb DNA Ladder (New England BioLabs Inc., Ipswich, MA) and 3 μL of 10 X loading buffer combined with 27 μL of DNA product (25 $\text{ng.}\mu\text{L}^{-1}$) and electrophoresed in 1 X Tris-acetate buffer (TAE) at 100 V for 60 min. Bands were visualized with a MP 4+ Instant Camera System (Polaroid, exposure 2, aperture 5.6).

RESULTS AND DISCUSSION

Inconsistencies between DNA isolation methods make phylogenetic comparisons between cloned libraries of 16S rRNA genes from rumen samples difficult. Fresh rumen samples that are untreated and frozen for later analysis undergo rapid DNA degradation when thawed. Our method yields bacterial pellets that may be preserved frozen in ASL buffer for processing at a later date. This protects the samples from DNA degradation when the rumen bacterial samples are subsequently thawed. Bacterial DNA that is released from lysed cells during the freeze/thaw cycle are protected and captured in the ASL buffer solution.

A recent method that was used to isolate particulate associated bacteria from feed particles for subsequent DNA extraction utilized Tween 80 in the processing protocol (8). Tween 80 has been shown to lyse bacterial cells at low concentrations, especially *Fibrobacter succinogenes* (9), an important cellulolytic rumen bacterium. Indeed, the method of Larue *et. al* (8) resulted in a 16S rDNA library that was devoid of *Fibrobacter* related clones. Our method uses an alternate methylcellulose and agitation procedure to release particulate attached bacteria. The method results in 16S rRNA gene libraries prepared from dairy cattle rumen samples that includes sequences related to the fragile *Fibrobacter succinogenes* (data not shown).

Our method of isolation is designed to dissociate bacteria from the most fibrous component of digesta in herbivores. Changes in diet and the physical composition of the digesta may require small changes in the protocol for rinsing and removal of liquid and particulate bacteria. Diets with low fiber content may have proportionately larger

amounts of liquid associated bacteria to remove with phosphate buffer. Diets with different fiber matrices found in wild forages such as lichens or browse may require modifications in the time and homogenization for methylcellulose release.

Adding mutanolysin and proteinase K to the extraction protocol yields additional DNA that would otherwise not be recovered (Fig. 3.1). The traditional QIAamp DNA Stool Mini Kit (Qiagen, Mississauga, ON, Canada) method fails to enzymatically release rumen bacterial DNA to obtain the maximum quantity of recoverable DNA. Addition of two enzymes to the extraction and using a bead-beating step increased DNA yield by 63%. Prior to adding mutanolysin and proteinase K, PCR-quality DNA concentrations were $32.0 \pm 6.1 \text{ ng.}\mu\text{L}^{-1}$ (mean \pm SD, $n = 3$) whereas the modified extraction produced concentrations of PCR-amplifiable DNA that were $52.6 \pm 7.1 \text{ ng.}\mu\text{L}^{-1}$. It should be noted that unlike other bead-beating procedures, our method does not require extraction with phenol or chloroform, reducing exposure to these chemicals and reducing the time and labor needed to process the samples.

The 16s rDNA PCR amplification procedure was sensitive to the concentrations of MgCl_2 and BSA within the reaction mixture. PCR amplification did not occur with standard concentrations of these, reaction components; however, the reaction was completed when the concentration of 5X PCR buffer was increased two-fold (50 mM Tris, pH 8.3, 2.5 mM Invitrogen MgCl_2 , 500 $\mu\text{g.mL}^{-1}$ Biolabs BSA). .

DNA extraction protocols for rumen bacteria have important applications to microbial ecosystem dynamics, diversity estimates, and molecular ecology. The modified procedure will better aid researchers in determining rumen microbial

phylotypes (10) by allowing recovery of DNA from bacterial cells in heterogeneous media.

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Table 3.1. Concentrations for amplifying 16s rDNA by PCR with a total reaction volume of 200 μL .

Component	Final Concentration	Concentration of Stock	Volume of Stock
5 X Buffer			80 μL
dNTP's	500 μM each	10 mM	10 μL
Forward primer	800 nM	10 pM, μL^{-1}	16
Reverse primer	800 nM	10 pM, μL^{-1}	16
DNA Polymerase	20 U	5 U, μL^{-1}	4
H ₂ O			70
DNA Template			4

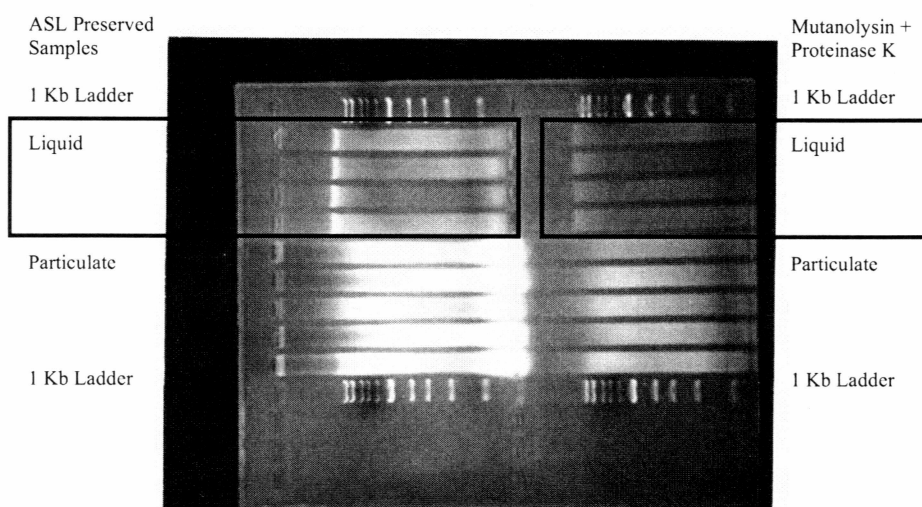


Figure 3.1. Stepwise extraction of 16S rDNA from rumen bacteria visualized by ethidium bromide staining on a 10% TAE agarose gel. Supernatants from the rumen samples frozen in ASL buffer and processed with QIAamp DNA Stool Mini Kit (left). Further extraction with mutanolysin + proteinase K and bead-beating (right) yielded additional DNA from both liquid and particulate fractions.

CONCLUSIONS

Seasonal changes in substrate load within the rumen of muskoxen appear to drive shifts in rumen environmental parameters as well as microbial activity. Ruminal conditions of temperature, osmolality, and pH were affected by the quantity of substrate available for microbial fermentation. Concentrations of bacteria within the rumen likely follow patterns of food intake in muskoxen as declines in bacterial numbers over winter were consistent with drops in water turnover rates. Brief drops in rumen temperature, however, did not appear to affect microbial dynamics. Despite variable temperature minima, the maximum upper limit of rumen temperature is probably tightly regulated as temperatures never exceed 41°C. Overheating within the rumen could substantially impair the ability of the microbes to degrade fiber.

Despite large thermal differences between ambient conditions in the high arctic and rumen temperatures in muskoxen, the cost of ingesting food was not energetically unfavorable to the animal. Although the thermal cost of warming ingesta during early, mid, and late winter was 2.7, 14.2, and 10.3% of the digestible energy intake, respectively, estimated heat increments of feeding exceeded the costs associated with ingesting both cold water and frozen forage. Therefore, natural decreases in food consumption by muskoxen during the coldest winter months in January and March appear to limit the potentially taxing costs required to warm ingesta.

Regulation of rumen fermentation during periods of low and high food intake revealed varying ruminal conditions between May and August, respectively. Increased microbial activity during August, when food intakes are high, created greater

concentrations of fermentation products. Moreover, with the challenge of an acute load of fermentable substrate was provided to the system during May and August, the microbial response increased SCFA and decreased rumen fluid pH appropriately. However, greater variability in the rumen environment with respect to pH and bacterial numbers when food intakes are low in muskoxen may indicate that parameters within the microbial ecosystem are more tightly controlled in August. High food intakes by the host animal increase blood flow to the rumen and absorption by the mucosa, enabling the rumen to quickly respond to high substrate loads and return to pre-existing conditions.

Seasonal changes in food intake by muskoxen cause shifts in rumen conditions as well as microbial activity. The rumen ecosystem likely possesses great diversity of microbial expression in order to accommodate these broad fluctuations in environmental parameters. However, estimating this bacterial diversity has been limited in the past by the ability to extract equal proportions of DNA from both phases of rumen digesta: liquid and particulate. The alternative DNA isolation and extraction protocol created by modifying the existing QIAamp DNA Stool Mini Kit yields 63% more rumen bacterial DNA. By utilizing more accurate methodology for 16s rDNA bacterial diversity estimates, phylogenetic relationships that exist within the rumen microbial ecosystem of muskoxen may be ascertained.